R53-49

Reprinted from APPLIED MICROBIOLOGY
Vol. 1, No. 1, 1953
Printed in U.S.A.

The Microbiological Degradation of Plasticizers

I. Growth on Esters and Alcohols

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Received for publication August 20, 1952

The production of thin films of synthetic plastics in the form of coating materials and unsupported films has led to the development of a major industry within the last ten years. The use of these films was made possible by compounding vinyl resins with a plasticizer, usually a high-molecular weight ester, in order to impart a desired degree of flexibility to the otherwise hard and brittle plastic. However, under trial during the extremely varied conditions of global warfare such plasticized resins revealed shortcomings, one of which was a frequent localized stiffening of the film traceable to the growth of fungi or bacteria, or both. It was shown that it is the plasticizer, and not the plastic itself, that is attacked by these microorganisms (Molnar and Leonard, 1945; Wellman and McCallan, 1945); the observed embrittlement of the plastic composition was thus explained by the progressive disappearance of plasticizer. The findings of the above and many other authors have been summarized in an O.S.R.D. report on tropical deterioration (Brown, 1945), and more recently a survey of development in films and coated fabrics by the Quartermaster Corps has been published (Boor, 1950). It was of interest to investigate this degradation further, with a view to elucidating its mechanism with respect to the relationship between molecular structure of related series of plasticizers and their ability to support growth of fungi and bacteria.

For the present purpose we shall adopt the classification of monomeric plasticizers in two broad categories: "fatty oil"-type plasticizers, including castor oil (glyceryl triricinoleate) coconut oil, or cottonseed oil derivatives; and, "non-fatty oil"-type plasticizers, including alkyl esters of phosphoric, phthalic, tricarboxylic (e.g., citric and aconitic), and dicarboxylic (e.g., adipic and sebacic) acids. "Fatty oil"-type plasticizers are considerably cheaper and in better supply than the "non-fatty oil"-type and, in addition, are effective in imparting desirable low-temperature flexibility. Their main disadvantage, as a group, is a pronounced susceptibility to microbial degradation, with consequent loss of flexibility of the compounded plastic. Esters of aliphatic dicarboxylic acids are less easily degraded, and phosphates, phthalates and citrates are known to be resistant.

There appears to be no available information on the relative prevalence of fungal versus bacterial degradation, although it can be assumed that environmental factors are of great importance in determining the type of growth. A recent study in these laboratories (Reese, Cravetz and Mandels, *in press*) has shown that the ability to degrade certain plasticizers is very common in fungi. Of 350 species of fungi tested on several typical plasticizers, 90 per cent grew on methyl acetyl ricinoleate and 60 per cent grew on dihexyl sebacate. In the present work both a fungus, *Aspergillus versicolor*, and a bacterium, *Pseudomonas aeruginosa*, were used. These organisms have been repeatedly isolated from degraded plastic films (Harvey, 1947, 1949).

The data here reported comprise the first of a series of at least four such articles which shall appear under the above general heading.

MATERIALS AND METHODS

Materials

Microorganisms. The fungus *Aspergillus versicolor*, QM 432 was carried as stock culture on Bacto potato dextrose agar; the bacterium *Pseudomonas aeruginosa* QMB 1468 was carried on Bacto nutrient agar.

Substrates. A large number of plasticizers were obtained as commercial samples. A series of homologous dialkyl sebacates, C_1–C_{18}, were synthesized, purified and characterized in this laboratory (Stahl and Pessen, 1952); alcohols, for the most part, were C. P. grade. Technical grades were redistilled and fractionated before use as substrates.

Methods

The ease of utilization of a particular substrate was evaluated by a quantitative measure of the growth of fungus or bacteria on it. Substrates (3 per cent in the case of all esters and 2 per cent for all alcohols) were weighed or pipetted into 250 ml Erlenmeyer flasks to which was added 70 ml of a mineral salts solution (100 ml stock A[1] + 100 ml stock B[2] made up to 1 liter; pH 6.6). Bacto yeast extract was also added to a final concentration of 1–10,000. The flasks were plugged with

[1] Stock soln. A = 30.0 g NH_4NO_3, 22.2 g $MgSO_4 \cdot 7H_2O$—to 1 liter.

[2] Stock soln. B = 25.9 g KH_2PO_4, 22.1 g K_2HPO_4—to 1 liter.

cotton and sterilized.[3] Those substrates having boiling points below 180 C were filtered through Seitz bacterial filters and added to the previously sterilized mineral salts solution and yeast extract just before inoculation. After inoculation, the flasks were fastened to a horizontal shaker reciprocating about 90 times per minute, with a stroke of 3 in; incubator temperature was 30 C. The incubation time was 7 days, except where indicated otherwise.

Since most of the esters and alcohols formed emulsions or dispersions, visual ratings of fungal growth and more particularly bacterial growth would have been difficult to make; therefore actual cell weights were determined after solvent extraction of the water-immiscible substrates.

To obtain the weight of fungi, the water-plasticizer mixture or emulsion containing the mycelial pellets was filtered through previously tared, wetted Soxhlet thimbles. The thimble contents were washed successively with acetone, water, 0.1 N hydrochloric acid and water again, extracted with acetone in a Soxhlet extractor for 4 hours, and dried to constant weight.

In the case of the bacteria, a turbidimetric procedure was established. The contents of a flask were exhaustively extracted with ethyl ether in a separatory funnel, the bacteria remaining entirely in the aqueous phase. The total volume of this phase was measured and its turbidity determined in a Klett-Summerson photoelectric colorimeter. The value thus obtained was converted into dry cell weight by means of a standard curve prepared for this purpose.

Results

Because organisms contain varying amounts of lipides, depending on the organism and on the nutrition supplied, the acetone extraction used here resulted in dry weights low by the amount of the organism's lipides, which, under the processing conditions employed, were extracted along with the ester. Typical contents of acetone-extractable constituents in *A. versicolor* and *P. aeruginosa* were found to be of the order of 20 per cent and 15 per cent, respectively, of dry weight. An error of this approximate magnitude may therefore be attached to the weights tabulated. No correction for it has been attempted because its exact evaluation, in view of the large number of samples run, would have been disproportionally laborious and presumably would have had little relevance to the relative rating of the various esters as growth substrates.

With this type of growth experiment it is, at best, difficult to obtain a high degree of reproducibility between experiments repeated at different times. Among the possible factors contributing to this variability are the size of the inoculum and the concentration of substrate. The effect of the former, shown in figure 1, was minimized by the use throughout of inocula resulting in spore concentrations of at least 3 million per flask. The effect of substrate concentration in the case of a typical plasticizer, methyl acetyl ricinoleate, is shown

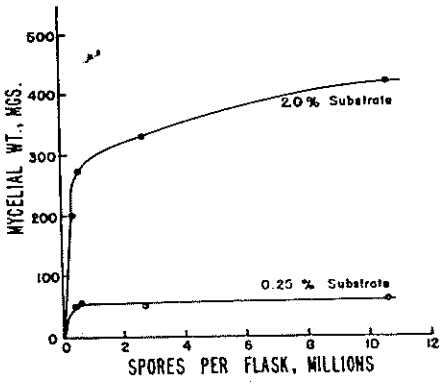

Fig. 1. Effect of variation of spore inoculum on mycelial growth. Grown at 30 C for 7 days.

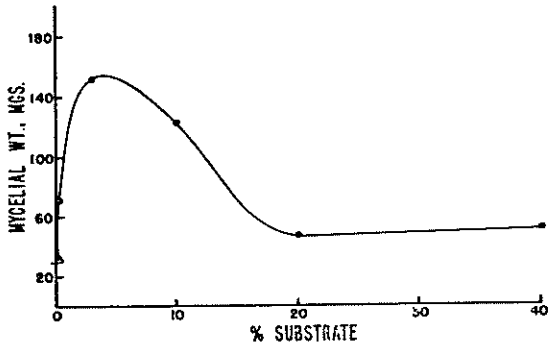

Fig. 2. Effect of variation of substrate concentration on mycelial growth. Grown at 30 C for 7 days.

in figure 2. For the most part 2 and 3 per cent substrate concentrations were employed. A definite effect on growth was observed for the extreme cases of tight and loose cotton plugs, respectively; plugs actually used were of approximately equal, medium tightness.

The degree of subdivision of the water-immiscible substrates might be expected to have an effect on the growth. Actually, even the more tacky, waxy solids were found to be kept well dispersed by constant agitation due to the shaker motion. While it is usual to add an emulsifying agent to insure more homogeneous mixtures, this practice was not adopted here. It is well known that surface-active agents are effective protein denaturants (Putnam and Neurath, 1944). Regardless of the presumed properties of the emulsifying or wetting agent and the low concentrations in which it is employed, its possible effects on the organism and on any elaborated enzymes would introduce an unpredictable

[3] In the case of substrates solid at room temperature it was found useful to allow solidification after sterilization to proceed by cooling the flask under a tap with constant swirling of the contents to avoid the conglomeration of the waxy ester into larger lumps.

TABLE 1. *Growth of microorganisms on various pure esters and commercial plasticizers serving as the only carbon source*

ESTER OR PLASTICIZER	*Aspergillus versicolor*, MGS.*		*Pseudomonas aeruginosa*, MGS.	
	Commercial product	Lab. synthesized product	Commercial product	Lab. synthesized product
Dimethyl sebacate	—	0	—	14
Diethyl sebacate	—	58	—	48
Di (n-propyl) sebacate	—	153	—	42
Di (n-butyl) sebacate	198	240	12	32
Di (n-amyl) sebacate	—	174	—	7
Di (isoamyl) sebacate	—	205	—	16
Di (3-methylbutyl) sebacate	—	143	—	10
Di (n-hexyl) sebacate	63	242	0	3
Di (1,3-dimethylbutyl) sebacate	—	27	—	3
Di (n-heptyl) sebacate	—	243	—	62
Di (n-octyl) sebacate	98	231	32	56
Di (2-ethylhexyl) sebacate	—	61	—	32
Di (2-methylheptyl) sebacate, (capryl)	62	240	9	16
Di (n-nonyl) sebacate	—	256	—	46
Di (n-decyl) sebacate	—	261	—	61
Di (n-undecyl) sebacate	—	136	—	68
Di (n-dodecyl) sebacate	—	111	—	71
Di (n-tridecyl) sebacate	—	96	—	61
Di (n-tetradecyl) sebacate	—	72	—	65
Di (n-pentadecyl) sebacate	—	26	—	60
Di (n-hexadecyl) sebacate	—	31	—	63
Di (n-heptadecyl) sebacate	—	26	—	55
Di (n-octadecyl) sebacate	—	33	—	50
Dibenzyl sebacate	256	171	—	13
Butyl benzyl sebacate	279	—	20	—
Di (n-decyl) adipate	—	240	—	62
Methyl ricinoleate	414	—	66	—
"Methyl cellosolve" ricinoleate	502	—	11	—
"Butyl cellosolve" ricinoleate	33	—	0	—
Butyl ricinoleate	385	—	13	—
Methyl acetyl ricinoleate	329	—	38	—
"Methyl cellosolve" acetyl ricinoleate	328	—	38	—
Butyl acetyl ricinoleate	154	—	37	—
Glyceryl ricinoleate	359	—	42	—
Glyceryl tri-acetyl ricinoleate	419	—	11	—
Butyl ester of acetylated polyricinoleic acid	187	—	0	—
Butyl octadecadienoate	111	—	46	—
Cyclohexyl ricinoleate	83	—	0	—
Triethylene glycol dicaprylate	154	—	32	—
Triethyl citrate	0	—	0	—
Tributyl citrate	0	—	—	—
Triethyl aconitate	0	—	—	—
3-hydroxy-2-ethylhexyl undecylenate	—	50	—	35
Tri (2-ethylhexyl) phosphate	0		3	—
Di (2-ethylhexyl) phthalate	0		4	—
Glycerol, C.P. (control), 2%	247	247	79	79
Sucrose, C.P. (control), 1%	227	227	8	8

* Figures in this and subsequent tables are averages of growth (after incubation for 7 days at 30 C) in 3 flasks, each containing 2.1 gm of the ester or 1.4 gm of the alcohol indicated, and 70 ml mineral salts and yeast extract solution.

additional variable as the price for obtaining at best a partial and not really imperative improvement in particle size.

Growth values reported in the following are generally averages of at least triplicates. With the majority of samples the average deviation from the mean was less than ±10 per cent and often much better, but in some cases it reached ±20 per cent. It was not found practicable to increase the reproducibility among replicates consistently to any material extent; while the data thus obtained cannot be used quantitatively without great caution, they were considered adequate to allow comparisons between significantly different substrates. All values listed (except those in figures 1 and 2) have been corrected for blanks (that is, the amount of growth shown by the regularly used amount of inoculum on 70 ml mineral salt solution with 0.01 per cent yeast extract alone). In the case of the fungus, an average blank in 9 groups of experiments was 37 mg per flask; in the case of the bacterium, an average of 10 groups was 2 mg per flask. A further aid in the interpretation of results is afforded by the observation, particularly with the alcohols, that while certain substrates showed no growth above that of the blank (i.e., they did not actively support growth), others showed no growth whatsoever, (i.e., they actively inhibited growth). Such instances are listed in the tables as "O (I)."

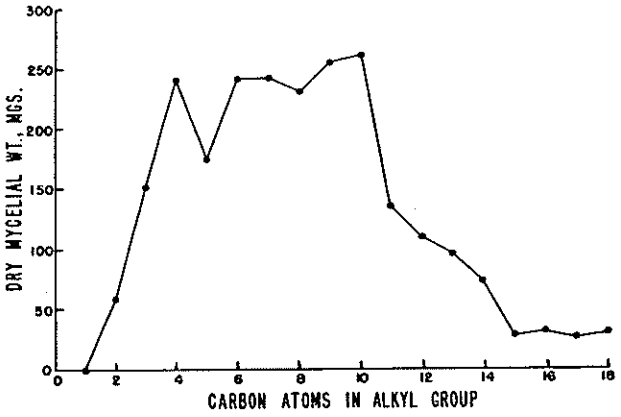

FIG. 3. Growth of *A. versicolor* on dialkyl sebacates as an only carbon source.

Values of pH after growth of *Aspergillus*, on either ester or alcohol substrates, varied from 6.6 (the initial value prior to inoculation due to the buffering effect of the mineral salts solution) to as low as 3.4. Generally a lowering of pH paralleled an increase in growth. An initial pH of 3 or below was inhibitory, the pH being optimal over a broad range from 6.2 to 8.0. Glycerol controls invariably dropped to pH 4.8.

The first half portion of table 1 lists the homologous series of sebacic acid diesters with alkyl radicals con-

taining from 1 to 18 carbons. It should be pointed out that among commercial plasticizers a number fall into this series, with 1 to 8 carbon alkyls. However, the homologous series in its entirety gives a more complete insight into the requirements for microbial growth. One notes that esters with alkyl radicals between C_3 and C_{10} readily support fungal growth, but of those which are solid even the C_{14} and C_{18} alkyl esters still support a small amount of growth. Only dimethyl sebacate supports no growth whatever. These values are illustrated in figure 3.

The second half of table 1 lists the fungal growth afforded by a representative group of commercial plasticizers. Using the "fatty oil" and "non-fatty oil" classification we note that plasticizers of the former type support growth without exception, whereas certain of

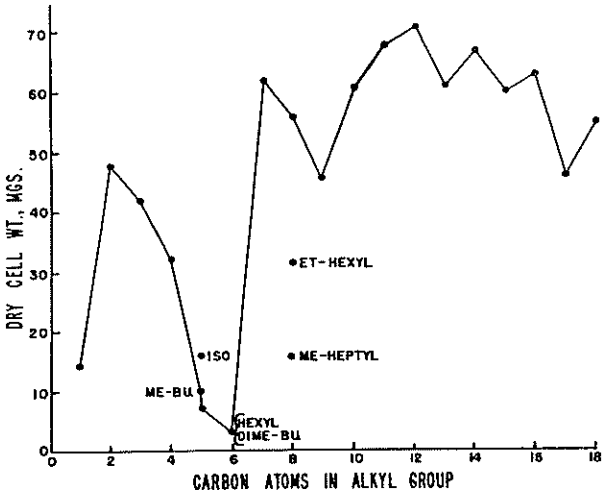

FIG. 4. Growth of *P. aeruginosa* on dialkyl sebacates as an only carbon source.

the latter type (specifically the phosphates, phthalates, and tricarboxylic acid esters) support no growth. This behavior shall be further discussed in a subsequent paper.

The corresponding values for the growth of *P. aeruginosa* are given in table 1 and figure 4. In contrast to figure 3, one notes quite different responses of the bacteria. While all of the esters support some growth there is a minimum at C_5–C_6 and a maximum around C_7, which persists, broadly speaking, out to C_{18}. The sharp minimum values at C_5 and C_6 at first were suspected as due to some gross error, but later were substantiated by another series of experiments. It is of interest to correlate this set of data with those obtained by Jezeski et al. (1950) by measuring O_2 consumption of species of 4 bacteria when incubated with methyl esters of saturated (even-numbered carbon) fatty acids. When μl O_2 consumed (the measure of growth) is plotted versus carbon atoms in the acid, curves somewhat similar to our figure 4 are obtained, where growth is plotted as a function of the number of carbon atoms in the alkyl group.

Discussion

Quantitative measurements of growth on ester and alcohol substrates were made to allow a comparison of their ease of conversion to actual cellular material. While this does not necessarily reflect the actual degree of degradation of plasticizer, as shall be shown in subsequent data, it affords some means of ranking plasticizers with respect to their susceptibility to microbiological degradation. Additionally, it serves as a biochemical criterion for characterizing the microorganisms. While it is recognized that the responses of *A. versicolor* and *P. aeruginosa* are not necessarily identical with those of other microorganisms, they may be thought of as typical of plasticizer-degrading microorganisms. Similarly, while the response to the substrates used here will differ from that toward other substrates, it may be considered as representative of certain broad classes of substrate.

The second half of table 1 shows that many "fatty oil" plasticizers do not support bacterial growth, and none do to the extent of that on the glycerol control. This is in rather sharp contrast to the data for the fungus. It would appear from this data that economically the problem of degradation by fungi might be the more important of the two, although in that respect environmental conditions probably will play a determinant part.

Some general similarities between the fungus and the bacterium are shown by the data of table 3 with regard to growth on alcohols, and by figures 3 and 4 with regard to growth on the homologous dialkyl sebacates. In spite of these similarities, inspection of the data makes evident distinct differences sufficient to illustrate the fact that the behavior of one type of microorganism need by no means parallel the behavior of another. This observation agrees with observed microbial responses toward toxic agents. Findings by the Chemicals and Plastics Division of Quartermaster Research and Development Laboratories (unpublished data) indicate that a plasticized resin containing amounts of fungicide adequate to prevent any fungal growth remains suceptible to bacterial attack, and that the converse is also true for the addition of bactericide. For adequate protection of a plastic film containing susceptible plasticizer both a fungicide and bactericide must therefore be included in the formulation.

Before the start of this work it was suggested by several manufacturers of plasticizers that the pure esters of the homologous series whose laboratory synthesis was then projected (Stahl and Pessen, 1952) would not support as much growth of microorganisms as their corresponding commercial products which are not highly

refined. A comparison of the relevant data indicates the reverse to be more nearly true with respect to the fungus, for example, see di(n-butyl), di(n-hexyl), di(n-octyl) and dicapryl sebacates; commercial products apparently either contain amounts of substances (possible free fatty acids or alcohols) sufficient to exert an inhibitory action, or the presence of isomeric alcohols in the ester has this same effect. With respect to the latter, it is known that a commercial product labeled "dioctyl sebacate" is mostly the 2-ethylhexyl ester,[4] since it has a melting point of −55 C, whereas the laboratory-prepared di(n-octyl) sebacate melts at +18.6 C. The bacteria apparently do not have the same powers of discrimination as the fungi, although there is a trend towards better growth on the purer product.

TABLE 2. *Effect of chain-branching of the plasticizer molecule on its availability as a carbon source to microorganisms*

PLASTICIZER		DRY WEIGHT, MGS.	
Component acid	Component alcohol	*A. versicolor*	*P. aeruginosa*
Sebacic	n-Amyl	174	7
	Isoamyl	205	16
	Methylbutyl	143	10
	n-Hexyl	242	3
	Dimethylbutyl	27	3
	n-Octyl	231	56
	Methylheptyl	256	16
	Ethylhexyl	61	32
Phthalic	n-Octyl	0	6
	Ethylhexyl	0	2
	Methylheptyl	0	3
Glycerol (control)		291	77

The above observations lead to an examination of the effect of chain-branching of a plasticizer molecule on its availability as a carbon source. Some of the data taken from table 1 is compared in table 2. While the effect on the fungus is not unequivocal, the trend is to show that an increase in chain-branching makes the compound less available to the organism for conversion to cellular mass. This is in apparent contrast to a finding (Peck and Rosenfield, 1938) that increase in chain-branching of fatty acids results in lessened fungicidal action, i.e. increased growth. Kitajima and Kawamura (1931) on the other hand observed no relationship between branching of the fatty acid chains and toxicity, but it must be noted that their growth conditions differed from those of Peck and Rosenfield. Again, the bacterium studied herein does not appear to discriminate. The differences in growth supported by plasticizers with isomeric alkyl groups may, at least in part, be due to the relatively lower solubilities of the branched esters, while the amounts of the isomeric acids used by the above cited workers are all water soluble. In toxicity studies, furthermore, the substance tested for inhibitory effect usually is added to a medium complete for the growth of organism. It should be emphasized that results obtained in this fashion are not directly comparable to those reported here, where the substances investigated constituted the only carbon source.

[4] This is not meant to be a reflection on any manufacturer's product whatsoever; it is realized that there must be a practical limit to the complexity of naming of a product.

TABLE 3. *Growth of microorganisms on various alcohols serving as the only source of carbon*

ALCOHOL	*Aspergillus versicolor*		*Pseudomonas aeruginosa*	
	Dry mycelial wt.	Incubation time	Dry cell wt.	Incubation time
	Mgs.	*Days*	*Mgs.*	*Days*
Glycerol	363	7	46	7
Ethylene glycol	50	7	0 (I)	23
Methyl "Cellosolve"	0	7	0 (I)	23
Butyl "Cellosolve"	0 (I)*	27	0 (I)	23
2-Ethylhexandiol-1,3	0	27	0 (I)	23
Triethylene glycol	12	7	0 (I)	7
Cyclohexanol	0 (I)	27	0 (I)	23
Benzyl alcohol	0 (I)	27	0 (I)	23
Ethyl-n-hexanol	0 (I)	27	0 (I)	23
Methyl-n-hexylcarbinol	0 (I)	27	0 (I)	23
Methyl alcohol	0	7	0 (I)	23
Ethyl alcohol	95	7	0	23
Propyl alcohol	0 (I)	27	0	23
Butyl alcohol	0 (I)	47	0	23
Amyl alcohol	0 (I)	27	0 (I)	23
Hexyl alcohol	0	18	0 (I)	23
Heptyl alcohol	0 (I)	27	0 (I)	23
Octyl alcohol	0 (I)	27	6	7
Nonyl alcohol	0 (I)	60	6	7
Decyl alcohol	0 (I)	27	7	7
Undecyl alcohol	0 (I)	56	44	7
Dodecyl alcohol	26	7	98	7
Tridecyl alcohol	116	7	67	7
Tetradecyl alcohol	104	7	58	7
Pentadecyl alcohol	110	7	58	7
Hexadecyl alcohol	101	7	54	7
Heptadecyl alcohol	—	—	51	7
Octadecyl alcohol	36	7	47	7

* The term 0 (I) refers to inhibition of any growth whatsoever. See text for discussion.

The most commonly used phthalate plasticizer is probably the di(2-ethylhexyl) phthalate, and since this branched-chain compound supported no growth, it was of interest to establish whether the di(n-octyl) compound would. Part II of table 2 shows that 3 isomers of the octyl ester support no fungal growth; all support a small amount of bacterial growth.

Comparing the growth of *A. versicolor* to *P. aeruginosa* on alcohols, as shown in table 3, one observes the following: while the glycols (ethylene and triethylene) support growth of fungus, they inhibit growth of the

bacteria; ethyl alcohol is a moderately good carbon source for the fungus, but inhibits growth of the bacteria; the fungus shows growth from C_{12} to C_{18}, whereas the bacteria begin to show growth from C_8 up. All of these alcohols are components of the esters studied. It may be noted that time of incubation is given in this table. It has been our experience that both fungus and bacteria when incubated with plasticizer at times showed no growth for perhaps 14 days and then suddenly multiplied rapidly. On examination, no evidence of contaminant organisms was found. Undoubtedly, there is a period of adaption of enzyme systems. However, when no growth is evidenced in 23 days or more on a particular substrate, the chances are exceedingly good that the organism is not going to adapt to growing on it.

A chance contamination of a group of plasticizers, presumably inoculated with known fungal spores, showed growth in pure culture of a yeast or yeast-like organism. It was then subcultured and later identified by Dr. H. J. Phaff, of the University of California, as *Zygosaccharomyces drosophilae* El Tabey (ined). Similarly to *Aspergillus versicolor*, this organism grew well on dihexyl sebacate, methyl ricinoleate, glyceryl triacetyl ricinoleate, triethylene glycol dicaprylate and olive oil. It did not grow on triethyl citrate, trioctyl phosphate or dioctyl phthalate. Subsequent experiments with a known culture of *Saccharomyces cerevisiae* exhibited the same growth or non-growth characteristics on the above substrates as *Z. drosophilae*, with one exception. *Z. drosophilae* grew well on triethylene glycol dicaprylate but *S. cerevisiae* did only slightly. Thus yeasts probably must be recognized as another potential group of plasticizer destroyers.

Acknowledgment

The authors wish to express their sincere thanks to Mr. L. Boor of the Chemicals and Plastics Division of Quartermaster Research and Development Laboratories and also to Dr. Claude F. Alexander of B. F. Goodrich Chemical Company, for their many valuable suggestions throughout the course of this investigation. We also extend our thanks to Mary V. Hannigan for preparing the tri-, penta- and heptadecyl alcohols used herein.

Summary

Aspergillus versicolor and *Pseudomonas aeruginosa* have been utilized as typical plasticizer-degrading organisms. Probably because of the presence of isomers or other impurities, commercial products often support less growth than pure, laboratory synthesized plasticizers, particularly in the case of the fungus. Responses of the fungus and the bacterium differ considerably as shown by tests on a variety of substrates, including a homologous series of dialkyl sebacates, a comprehensive group of commercial plasticizers, and the alcohols which are constituents of all these esters.

With the homologous sebacates, *A. versicolor* grew most readily on the esters with C_3 to C_{10} alkyls, whereas *P. aeruginosa* grew best on those with C_7 to C_{18} alkyls. *A. versicolor* grew on all fatty-oil plasticizers, *P. aeruginosa* showed no growth on some of them. *A. versicolor* grew on alcohols from C_{12} up, *P. aeruginosa* on those from C_8 up. Chain-branching of plasticizers decreased the growth of *A. versicolor* but was of little effect on *P. aeruginosa*. On the basis of these differences the general observation is made that a structural modification which renders a plasticizer fungus resistant will not necessarily make it bacteria resistant, or *vice versa.*

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